

CONJUGATES OF TC COMPLEXES AND TARGETING MOIETIES AND THEIR USE IN MRI DIAGNOSTIC

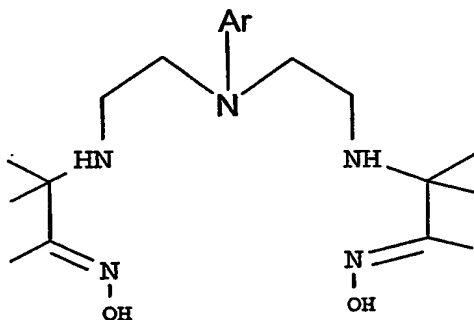
Field of the Invention.

- 5 The present invention relates to improved ^{99m}Tc radiometal complex compositions, radiopharmaceutical preparations containing them, together with kits for the preparation of the radiopharmaceuticals.

Background to the Invention.

- 10 US 5395608 discloses a range of potentially pentadentate aza-diaminedioxime ligands for the preparation of radiometal complexes of a wide range of radioisotopes, including ^{99m}Tc , ^{105}Rh , ^{109}Pd , ^{57}Co , ^{186}Re , ^{188}Re , ^{97}Ru , ^{111}In , ^{113m}In , ^{67}Ga and ^{68}Ga . Bifunctional aza-diaminedioxime ligands with pendant aryl spacer groups are disclosed, and Example 3 of US 5395608 describes the compound of Formula A:

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Formula A

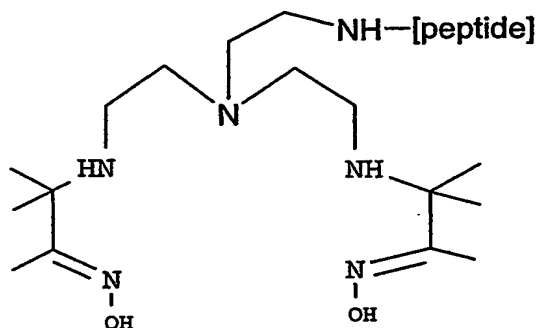
where Ar = 4-aminobenzyl.

US 5395608 describes the radiolabelling of such chelators in broad terms, but does not provide any specific Example on labelling with the radiometal ^{99m}Tc .

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Pillai *et al* [J.Nucl.Med.Biol., 38, 527-31(1994)] describe the ^{99m}Tc labelling of a aza-diaminedioxime analogue of Formula A, where Ar is benzyl. The ^{99m}Tc labelling is carried out at room temperature, and the HPLC traces show only a single ^{99m}Tc species. Conjugates of the aza-diaminedioxime with targeting molecules are not described.

WO 99/60018 discloses chelate conjugates of aza-diaminedioxime ligands with peptides for thrombus imaging. The peptides are substrates for the enzyme transglutaminase (ie. Factor XIIIa). A preferred such chelator conjugate is said to be an aza-diaminedioxime of formula:



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where the peptide is a fragment of α_2 -antiplasmin or casein.

The Present Invention.

It has now been found that the technetium metal complexation chemistry of such aza-diaminedioxime-targeting molecule chelator conjugates can give rise to a multiplicity of technetium species. The present invention provides an improved aza-diaminedioxime conjugate technetium metal complex composition in which the less desirable technetium species are suppressed.

Thus, aza-diaminedioxime chelator conjugates have been found to form several technetium species on chelation with ^xTc (where $x = 94\text{m}, 99$ or 99m). These multiple ^xTc metal complex species can be separated and detected by chromatography. Some of the multiple technetium species are kinetic products, ie. transient species which are converted over a period of time (optionally with heating) to give a stable product. The rate of conversion from the kinetically favoured (transient) complexes to the final specie is pH dependant: 2-3 hours are required at pH 9.5, whereas 60 minutes is usually sufficient at pH 10.5. The reaction can be accelerated by heating. It has also been found that some of these multiple ^xTc complex species are lipophilic, and can adversely effect the biodistribution of the radiopharmaceutical. . The present invention provides a technetium complex composition in which the level of these kinetic and lipophilic species

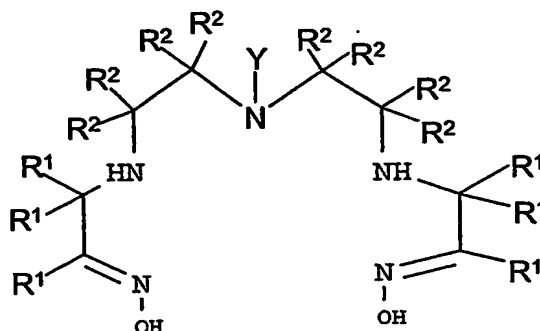
is minimised and controlled, hence improving the overall imaging characteristics. The present invention also provides methodology for optimising the conversion to the desired stable product, so that a controlled ^{99m}Tc complex product composition can be prepared and quality-controlled in a more rapid and reproducible manner.

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The present invention also provides radiopharmaceuticals comprising the improved technetium complex composition, together with non-radioactive kit formulations for the preparation of such radiopharmaceuticals.

10 Detailed Description of the Invention.

In a first aspect, the present invention provides, a technetium complex composition which comprises a metal complex of the radioisotope ^{99m}Tc with a ligand of Formula (I):



(I)

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where:

each R^1 and R^2 is independently an R group;

x is 94m, 99 or 99m;

Y is $-(A)_n-Z$

where: Z is a biological targeting moiety of molecular weight less than 5,000;

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$-(A)_n-$ is a linker group where each A is independently

$-\text{CO}-$, $-\text{CR}_2-$, $-\text{CR}=\text{CR}-$, $-\text{C}\equiv\text{C}-$, $-\text{CR}_2\text{CO}_2-$, $-\text{CO}_2\text{CR}_2-$, $-\text{NR}-$, $-\text{NRCO}-$, $-\text{CONR}-$, $-\text{NR}(\text{C}=\text{O})\text{NR}-$, $-\text{NR}(\text{C}=\text{S})\text{NR}-$, $-\text{SO}_2\text{NR}-$,

-NRSO₂- , -CR₂OCR₂- , -CR₂SCR₂- , -CR₂NRCR₂- , a C₄₋₈ cycloheteroalkylene group, a C₄₋₈ cycloalkylene group, a C₅₋₁₂ arylene group, or a C₃₋₁₂ heteroarylene group or a polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;
5 n is an integer of value 0 to 10;

each R group is independently H or C₁₋₁₀ alkyl, C₃₋₁₀ alkylaryl, C₂₋₁₀ alkoxyalkyl, C₁₋₁₀ hydroxyalkyl, C₁₋₁₀ fluoroalkyl, or 2 or more R groups, together with the atoms to which they are attached form a carbocyclic, heterocyclic, saturated or unsaturated ring;

10 wherein less than 10 % of the ^xTc present in the technetium complex composition comprises transient ^xTc complexes of the ligand of Formula I.

Suitable radioisotopes ^xTc of the present invention are therefore the positron emitter ^{94m}Tc, the beta emitter ⁹⁹Tc, or the γ-emitter ^{99m}Tc. Preferably, x is 99m, ie. the
15 radioisotope is preferably ^{99m}Tc.

By the term "ligand" is meant the conjugate of the aza-diamine dioxime chelator with the biological targeting molecule (Z). The ligand is designed to form aza-diamine dioxime metal complexes of the radioisotope ^xTc conjugated to the biological targeting molecule.

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By the term "biological targeting moiety" is meant: 3-100 mer peptides or peptide analogues which may be linear peptides or cyclic peptides or combinations thereof; or enzyme substrates or inhibitors; synthetic receptor-binding compounds; oligonucleotides, or oligo-DNA or oligo-RNA fragments. The biological targeting moiety may be of
25 synthetic or natural origin, but is preferably synthetic. Suitable biological targeting moieties of the present invention have a molecular weight less than 5,000 Daltons, preferably in the range 200 to 3000 Daltons, most preferably in the range 300 to 2000 Daltons, with 400 to 1500 being especially preferred.

30 By the term "cyclic peptide" is meant a sequence of 5 to 15 amino acids in which the two terminal amino acids are bonded together by a covalent bond which may be a peptide or

disulphide bond or a synthetic non-peptide bond such as a thioether, phosphodiester, disiloxane or urethane bond.

By the term "amino acid" is meant an *L*- or *D*-amino acid, amino acid analogue or amino acid mimetic which may be naturally occurring or of purely synthetic origin, and may be optically pure, i.e. a single enantiomer and hence chiral, or a mixture of enantiomers. Preferably the amino acids of the present invention are optically pure. By the term "amino acid mimetic" is meant synthetic analogues of naturally occurring amino acids which are isosteres, i.e. have been designed to mimic the steric and electronic structure of the natural compound. Such isosteres are well known to those skilled in the art and include but are not limited to depsipeptides, retro-inverso peptides, thioamides, cycloalkanes or 1,5-disubstituted tetrazoles [see M. Goodman, *Biopolymers*, 24, 137, (1985)].

Suitable enzyme substrates or inhibitors include glucose and glucose analogues such as fluorodeoxyglucose, fatty acids or elastase inhibitors. Suitable enzyme substrates or inhibitors may be biological targeting peptides (as described below).

Suitable synthetic receptor-binding compounds include estradiol, estrogen, progestin, progesterone and other steroid hormones; ligands for the dopamine D-1 or D-2 receptor, or dopamine transporter such as tropanes; and ligands for the serotonin receptor.

Suitable biological targeting peptides of the present invention are 3-20 mer peptides (ie. peptides comprising 3 to 20 amino acids), preferably 4 to 15-mer, most preferably 5 to 14-mer such fragments. The peptides may be cyclic or linear or combinations thereof. The peptides may be of synthetic or natural origin, but are preferably synthetic. Such peptides include:

- somatostatin, octreotide and analogues,
- laminin fragments eg. YIGSR, PDSGR, IKVAV, LRE and KCQAGTFALRGDPQG,
- N-formyl peptides for targeting sites of leucocyte accumulation,

- fragments of platelet factor 4 (PF4),
- RGD-containing peptides,
- peptide fragments of α_2 -antiplasmin, fibronectin or beta-casein, fibrinogen or thrombospondin, which are substrates for the enzyme transglutaminase (Factor XIIIa). The amino acid sequences of α_2 -antiplasmin, fibronectin, beta-casein, fibrinogen and thrombospondin can be found in the following references: α_2 -antiplasmin precursor [M.Tone *et al.*, J.Biochem, 102, 1033, (1987)]; beta-casein [L.Hansson *et al.*, Gene, 139, 193, (1994)]; fibronectin [A.Gutman *et al.*, FEBS Lett., 207, 145, (1996)]; thrombospondin-1 precursor [V.Dixit *et al.*, Proc. Natl. Acad. Sci., USA, 83, 5449, (1986)]; R.F.Doolittle, Ann. Rev. Biochem., 53, 195, (1984).

When the biological targeting moiety is a peptide, one or both of the peptide termini may optionally be protected with a suitable metabolism inhibiting group. By the term “metabolism inhibiting group” is meant a biocompatible group which inhibits or suppresses *in vivo* metabolism of the peptide or amino acid at the amino or carboxyl terminus. Such groups are well known to those skilled in the art and are suitably chosen from, for the peptide amine terminus: acetyl, Boc (where Boc is *tert*-butyloxycarbonyl), Fmoc (where Fmoc is fluorenylmethoxycarbonyl), benzyloxycarbonyl, trifluoroacetyl, allyloxycarbonyl, Dde [i.e. 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] or Npys (i.e. 3-nitro-2-pyridine sulfenyl). Inhibition of metabolism of the peptide amine terminus may also be achieved by attachment of the amine terminus to the aza-diamine dioxime chelator. Suitable metabolism inhibiting groups for the peptide carboxyl terminus include: carboxamide, *tert*-butyl ester, benzyl ester, cyclohexyl ester, amino alcohol or attachment to the aza-diamine dioxime chelator. Preferably, at least one metabolism inhibiting group is chosen to be the aza-diamine dioxime chelator.

The carboxyl terminus of peptides is particularly susceptible to *in vivo* cleavage by carboxypeptidase enzymes. Consequently, the aza-diamine dioxime chelator is preferably attached at the carboxyl terminus. When the peptide comprises a cyclic peptide, the

metabolism inhibiting group may be the covalent bond which closes the cyclic peptide ring.

5 Preferred biological targeting peptides of the present invention are 3 to 20-mer peptide fragments of α_2 -antiplasmin or casein, most preferably 4 to 15-mer, especially 5 to 14-mer such fragments. Preferred α_2 -antiplasmin or casein peptides of the present invention comprise at least one metabolism inhibiting group, and an amino acid sequence taken from the N-terminus of either:

10 (i) α_2 -antiplasmin,

i.e. $\text{NH}_2\text{-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-OH}$
or variants of this in which one or more amino acids have been exchanged, added or removed such as:

$\text{NH}_2\text{-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-Gly-OH},$
15 $\text{NH}_2\text{-Asn-Gln-Glu-Ala-Val-Ser-Pro-Leu-Thr-Leu-Thr-Leu-Leu-Lys-Gly-OH},$
 $\text{NH}_2\text{-Asn-Gln-Glu-Gln-Val-Gly-OH};$ or

(ii) casein

ie. $\text{Ac-Leu-Gly-Pro-Gly-Gln-Ser-Lys-Val-Ile-Gly}.$

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Especially preferred α_2 -antiplasmin peptides of the present invention are peptide fragments comprising the 4 amino acid sequence Asn-Gln-Glu-Gln (NQEQ). Most especially preferred such α_2 -antiplasmin peptides have the sequence:

$\text{Asn-Gln-Glu-Gln-Val-Ser-Pro-Xaa-Thr-Leu-Leu-Lys-Gly},$

25 where Xaa is Tyr or I-Tyr (ie. iodo-tyrosine).

Such preferred α_2 -antiplasmin peptides preferably have metabolism inhibiting groups at both the peptide termini, where the aza-diaminedioxime is one of the metabolism inhibiting groups. In that instance, the aza-diaminedioxime chelator is preferably attached at the carboxy terminus, and the N-terminus is protected by a metabolism inhibiting
30 group, preferably N-acetyl so that the α_2 -antiplasmin peptide is preferably:

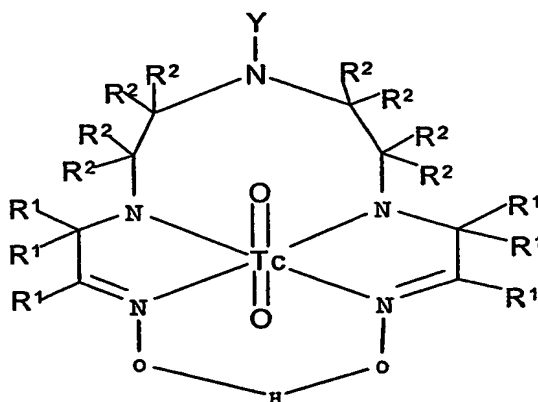
Ac-Asn-Gln-Glu-Gln-Val-Ser-Pro-Xaa-Thr-Leu-Leu-Lys-Gly,
where Xaa is Tyr or I-Tyr (ie. iodo-tyrosine).

By the term 'fluoroalkyl' is meant an alkyl group with at least one fluorine substituent, ie.
5 the term encompasses groups from monofluoroalkyl (eg. $-\text{CH}_2\text{F}$) to perfluoroalkyl (eg. CF_3).

The present inventors have found that, in addition to the desired technetium species, aza-
diaminedioxime ligands form other technetium complexes which are categorised as
10 transient $^{\text{Tc}}$ complexes of the ligand of Formula I. Technetium complex compositions of
the present invention are those characterised in that less than 10 % of the $^{\text{Tc}}$ present in
the technetium complex composition comprises transient $^{\text{Tc}}$ complexes of the ligand of
Formula I. These "transient $^{\text{Tc}}$ complexes" are the kinetically favoured technetium metal
complexation reaction products with the ligand of Formula I. They are transient in nature,
15 slowly converting to the desired thermodynamically stable final chemical complexation
reaction product under ambient conditions. The chemical structures of the transient $^{\text{Tc}}$
complexes are not known, but they are believed to involve coordination of the bridgehead
nitrogen atom of the $-\text{NY}-$ moiety of the ligand of Formula (I) to the technetium. Such
coordination would probably involve the aza-diaminedioxime ligand functioning either as
20 a pentadentate ligand (ie. having a dioximetriamine donor set), or as a tetradentate
chelator with one uncoordinated oxime group in a Tc(V) complex with a monoxo core (ie.
a $\text{Tc}=\text{O}^{3+}$ core). Such coordination by the bridgehead nitrogen atom would be kinetically
favoured by the smaller 5-ring chelate ring size when the $-\text{NY}-$ nitrogen atom
coordinates, compared to the 8-ring chelate ring size of the thermodynamic product
25 wherein the $-\text{NY}-$ nitrogen atom is uncoordinated.

For radiopharmaceutical applications in particular, the presence of multiple technetium
species whose exact composition changes with time is clearly undesirable, since
reproducibility of what is administered is difficult to achieve, and each species may have
30 different biodistribution characteristics. The present invention therefore provides methods
for controlling the levels of the transient technetium complexes, so that the desired

thermodynamic product is obtained in the highest degree of purity. It is believed that the desired thermodynamic product of the technetium complexes of the aza-diaminedioxime ligand has the structure:



where R^1 , R^2 and Y are as defined above;

- ie. a Tc(V) dioxo complex, with the aza-diaminedioxime ligand having a diaminedioxime metal donor set, where the nitrogen of the -NY- group is uncoordinated. The pseudo-macrocyclic ring structure is believed to confer thermodynamic stability compared with the transient $^{\text{Tc}}$ complexes. When the nature of Y is such that the N atom of -NY- is a tertiary amine, then a significant proportion of this tertiary amine is protonated in aqueous solution at pH less than 9.0 due to the basicity of tertiary amines (which have pK_a values typically in the range 8 to 10).
- The present inventors have found that, in addition to the desired technetium species, and the transient $^{\text{Tc}}$ complexes, aza-diaminedioxime ligands may form other technetium complexes which are categorised as lipophilic technetium complexes, and that such lipophilic complexes can adversely effect the biodistribution of the $^{\text{Tc}}$ composition. By the term "lipophilic technetium complex" is meant an aza-diaminedioxime metal coordination complex of technetium with the ligand of Formula I which has a significantly greater octanol water partition coefficient than the desired thermodynamic technetium complex of the aza-diaminedioxime. Such "lipophilic technetium complexes" bind strongly to the stationary phase in C18 reverse-phase HPLC when up to 50% organic

solvent is used in the mobile phase. Therefore the presence of such a "lipophilic technetium complex" in the composition necessitates the use of an aqueous/organic solvent mobile phase with an organic content of at least 70%, preferably at least 90% to cause them to elute from the column in a reverse-phase HPLC analysis.

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It is believed that such "lipophilic technetium complexes" are particularly likely to be observed when lyophilised ligands of Formula I are prepared in pharmaceutical vials or containers which comprise a closure made of synthetic elastomeric rubber, and the biological targeting molecule (Z) comprises a reactive nucleophilic group such as an amine-containing compound, especially one having a primary or secondary amine (eg. a Lysine residue of a peptide); a thiol (eg. a Cysteine residue of a peptide), or a phenol (eg. a Tyrosine residue of a peptide). In such circumstances, volatile non-polar leachables from the synthetic rubber may be removed from the synthetic closure *in vacuo*, and condense on the cold lyophilisation plug. Chemical reaction of the leached compound with the nucleophilic group of the targeting molecule then leads to the formation of more lipophilic derivatives of Z. Such chemical reactions are favoured at alkaline pH, where the nucleophilic group is relatively reactive (ie. free as opposed to protonated amines, or a greater degree of thiolate or phenolate species being present). When the ^{99m}Tc radiolabelling is carried out, the result is that "lipophilic technetium complex" impurities are observed. The leaching of a range of volatile, non-polar leachables from synthetic rubber closures during lyophilisation is known [see Jahnke *et al*, *Int.J.Pharmaceut.*, 77, 47-55 (1991) and *J.Parent.Sci.Technol.*, 44(5), 282- 288 (1990)], but the effect of these leachables on radiopharmaceutical agents has not previously been reported. It is also known that such closure leachables can arise during heating, eg. during autoclaving for sterilisation [see eg. Danielson *J.Parent.Sci.Technol.* 46(2), 43-47 (1992)], so it is believed that reaction of the biological targeting molecule (Z) with such leachables can occur in other circumstances also, although lyophilisation is the worst case scenario.

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Such "lipophilic technetium complexes" are therefore particularly problematic when the technetium complex composition is prepared in a vial or container having a synthetic closure, especially when lyophilisation is used - such as the kits of the third embodiment

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below. The present invention provides methods of suppressing or controlling "lipophilic technetium complexes" by suitable choice of closure materials and kit formulations.

Preferred technetium complex compositions of the present invention are those wherein, in addition to a suppressed level of transient ^{99m}Tc complexes, less than 5 % of the ^{99m}Tc present in the technetium complex composition comprises lipophilic ^{99m}Tc complexes as defined above. Preferably, technetium complex compositions of the present invention are those wherein less than 3 % of the ^{99m}Tc present in the technetium complex composition comprises lipophilic ^{99m}Tc complexes. Most preferably, the level of these lipophilic ^{99m}Tc complexes is less than 2 %, with less than 1 % being especially preferred, and less than 0.5 % being the ideal.

The present invention shows that the lipophilic ^{99m}Tc complexes of the ligand of Formula I exhibit undesirable liver uptake and blood retention *in vivo*. Minimising liver uptake is important, since the liver is an important background organ for radiopharmaceutical imaging – especially for lung or heart imaging. When the targeting molecule (Z) is designed to target thrombi within the lung such as pulmonary emboli (PE), the potential for imaging will also be affected by radioactivity in the organs and tissues surrounding the blood clot (i.e. lung, heart, blood and liver).

The various technetium aza-diaminedioxime complexes of the present invention can be separated and detected by suitable chromatographic methods, such as HPLC, especially reversed phase HPLC, ie. RP-HPLC. The presence of these lipophilic and kinetic species was not recognised by previous investigators. Thus, eg. initial HPLC and ITLC analysis of an aza-diaminedioxime conjugate with an antiplasmin peptide suggested a nominal purity of over 90 %, ie. a satisfactory purity. Subsequent biodistribution studies exhibited high levels of retention in the liver which were inconsistent with the measured amounts of radiochemical impurities. The impurities included RHT (reduced hydrolysed technetium), which is known to exhibit liver uptake. RHT is an immobile species which is detected by ITLC. Further more discriminating chromatographic analysis showed the multiple species of the present invention.

Using RP-HPLC, the transient ^{99m}Tc complexes exhibit retention times less than that of the thermodynamic product, whereas the lipophilic complexes exhibit significantly longer retention times than that of the thermodynamic product. Using quantification of peak size, the person skilled in the art can thus readily monitor whether a given ^{99m}Tc complexation reaction has proceeded to completion, and the levels of lipophilic ^{99m}Tc complexes present. An authentic sample of the desired thermodynamic ^{99m}Tc complex product can readily be established for a given system by either a prolonged complexation reaction time at ambient temperature (18-25 °C), or following heating at more elevated temperatures as taught by the present invention. Such authentic samples can then be used to identify the peaks observed for the particular chromatography system used.

The term "logP" has its' standard meaning – ie. the logarithm (in base 10) of the octanol-water partition coefficient (P). Such octanol-water partition coefficients are published for a wide range of organic compounds, and can be measured by a variety of techniques, such as direct measurement [for ^{99m}Tc complexes see A.R.Ketring *et al*, Int.J.Nucl.Med.Biol., 11, 113-119 (1984)], or chromatography [A.Hoepping *et al*, Bioorg.Med.Chem., 6, 1663-1672 (1998)]. A preferred measurement method is to calibrate a chromatography system (eg. HPLC) with known standards so that the retention time can be correlated with logP. This has the advantage that mixtures of species can be measured concurrently.

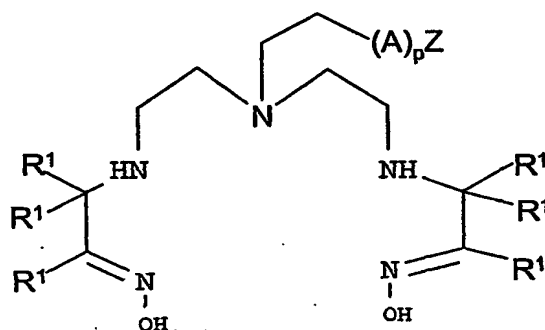
Preferred technetium complexes of the present invention are symmetrical, ie. the two $-\text{CR}^2_2\text{R}^2\text{NHCR}^1_2\text{C}(=\text{N}-\text{OH})\text{R}^1$ substituents on the $-\text{NY}-$ moiety of the ligand of Formula (I) are chosen to be the same. This has the advantage that, the technetium complex does not contain a chiral centre, since such centres may result in diastereomeric technetium complexes and possibly require the purification of particular isomers.

In the technetium aza-diamine dioxime complexes of the present invention, it is preferred that at least one R^2 group is H, more preferably all of the R^2 groups are H. Each R^1 is preferably chosen from: C_{1-3} alkyl, C_{2-4} alkoxyalkyl, C_{1-3} hydroxyalkyl, or C_{1-3}

fluoroalkyl, and is most preferably C₁₋₃ alkyl or C₁₋₃ fluoroalkyl. It is most especially preferred that all the R¹ groups are CH₃.

When the technetium complexes of the present invention comprise R groups wherein two or more R groups, together with the atoms to which they are attached, form a carbocyclic, heterocyclic, saturated or unsaturated ring, preferred such rings have 3- to 6-members, especially 5- or 6- members. Most preferred such rings are saturated carbocyclic rings. Preferred carbocyclic rings are those in which 2 R¹ groups attached to either the same or adjacent carbon atoms are combined to form 3- to 6-membered, especially 5- or 6-membered saturated rings.

Especially preferred technetium aza-diaminedioxime complex compositions of the present invention are those with the ligands of Formula (II):



II

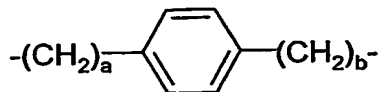
where each R¹ is independently chosen from C₁₋₃ alkyl or C₁₋₃ fluoroalkyl, p is an integer of value 0 to 3, and Z is as defined for Formula I. Thus, in Formula II Y is -CH₂CH₂(A)_pZ. Preferred ligands of Formula (II) are symmetrical as described above.

It is envisaged that the role of the linker group -(A)_n- is to distance the relatively bulky technetium complex which results upon metal coordination, from the active site of the biological targeting moiety (Z), so that eg. receptor binding is not impaired. This can be achieved by a combination of flexibility (eg. simple alkyl chains), so that the bulky group has the freedom to position itself away from the active site and/or rigidity such as a

cycloalkyl or aryl spacer which orientates the metal complex away from the active site. The nature of the linker group can also be used to modify the biodistribution of the resulting technetium complex of the conjugate. Thus, eg. the introduction of ether groups in the linker will help to minimise plasma protein binding, or the use of polymeric linker groups such as polyalkyleneglycol, especially PEG (polyethyleneglycol) can help to
5 prolong the lifetime of the agent in the blood *in vivo*.

Preferred linker groups $-(A)_n-$ have a backbone chain (ie. the linked atoms which make up the $-(A)_n-$ moiety) which contains 2 to 10 atoms, most preferably 2 to 5 atoms, with 2 or 3
10 atoms being especially preferred. A minimum linker group backbone chain of 2 atoms confers the advantage that the aza-diaminedioxime chelator is well-separated from the biological targeting moiety so that any interaction is minimised. A further advantage is that the potential chelate ring size of the Z group is so large (at least 8 for a 2 atom linker group chain), that these groups are unlikely to compete effectively with the coordination
15 of the aza-diaminedioxime to the ^{99m}Tc . In this way, both the biological targeting characteristics of the biological targeting moiety, and the metal complexing capability of the aza-diaminedioxime chelator is maintained in conjugates of this type. It is strongly preferred that the biological targeting moiety Z is bound to the aza-diaminedioxime chelator in such a way that the linkage does not undergo facile metabolism in blood. That
20 is because such metabolism would result in the ^{99m}Tc metal complex being cleaved off before the labelled biological targeting moiety reaches the desired *in vivo* target site. The biological targeting moiety is therefore preferably covalently bound to the ^{99m}Tc metal complexes of the present invention *via* $-(A)_n-$ linker groups which are not readily metabolised. Suitable such linkages are carbon-carbon bonds, amide bonds, urea or
25 thiourea linkages, or ether bonds.

Non-peptide linker groups such as alkylene groups or arylene groups have the advantage that there are no significant hydrogen bonding interactions with the conjugated biological targeting moiety so that the linker does not wrap round onto the biological targeting
30 moiety. Preferred alkylene spacer groups are $-(\text{CH}_2)_q-$ where q is an integer of value 2 to 5. Preferably q is 2 or 3. Preferred arylene spacers are of formula:



where: a and b are each independently 0, 1 or 2.

A preferred Y group is thus $-\text{CH}_2\text{CH}_2-(\text{A})_p\text{-Z}$, - where p is an integer of value 0 to 3.

5 When Z is a peptide, Y is preferably $-\text{CH}_2\text{CH}_2-(\text{A})_p\text{-Z}$ where $-(\text{A})_p\text{-}$ is $-\text{CO-}$ or $-\text{NR-}$. When $-(\text{A})_p\text{-}$ is $-\text{NH-}$, this grouping has the additional advantage that it stems from the symmetrical intermediate $\text{N}(\text{CH}_2\text{CH}_2\text{NH}_2)_3$, which is commercially available. Triamine precursors having different chain lengths require the use of synthetic strategies to chemically distinguish the various amines (eg. *via* protecting groups).

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The preferred aza-diaminedioxime chelators of Formula II are preferably conjugated to the $\alpha 2$ -antiplasmin peptides (and preferred embodiments thereof) described above.

15 The improved technetium complex compositions of the present invention may be prepared by reacting the ^xTc radioisotope (ie. $^{99\text{m}}\text{Tc}$, ^{99}Tc or $^{94\text{m}}\text{Tc}$) in the appropriate oxidation state with the ligand of Formula (I) or (II) in solution in a suitable solvent, with either:

- (i) a prolonged reaction time at ambient temperature; or
- (ii) heating at more elevated temperature; or
- 20 (iii) combinations thereof;

to drive the reaction to the desired thermodynamic ^xTc complex product, and thus minimise the level of the "transient ^xTc complexes". The usual technetium starting material is pertechnetate, ie. TcO_4^- which is technetium in the Tc(VII) oxidation state. Pertechnetate itself does not readily form metal complexes, hence the preparation of
 25 technetium complexes usually requires the addition of a suitable reducing agent such as stannous ion to facilitate complexation by reducing the oxidation state of the technetium to the lower oxidation states, usually Tc(I) to Tc(V) . The solvent may be organic or aqueous, or mixtures thereof. When the solvent comprises an organic solvent, the

organic solvent is preferably a biocompatible solvent, such as ethanol or DMSO. Preferably the solvent is aqueous, and is most preferably isotonic saline. To obtain the technetium complex compositions of the present invention, the reaction mixture must be heated at a minimum temperature of 50 °C for a period of at least 10 minutes. Suitable heating conditions are a temperature range of 50 to 80 °C for 10 to 20 minutes, preferably 60 to 70 °C for 10 to 15 minutes, most preferably 60 to 65 °C for 10 to 12 minutes, with 60 °C for 10 minutes being the ideal. The pH of the reaction medium is suitably in the range pH 8.5 to 9.5, preferably pH 8.8 to 9.0, most preferably pH 8.8. The heating process may employ any suitable methodology such as hot baths of fluid, such as water or a high-boiling oil (eg. silicone), heating blocks, hot plates or microwave radiation, as long as the desired temperature control can be achieved. After the heating is complete, the reaction mixture is either allowed to cool to room temperature, or may be actively cooled (eg. in a stream of a cooling fluid such as a gas or water) or *via* a heating block with integral inductive cooling.

The present inventors have also established that the undesirable lipophilic ^{99m}Tc complexes can be suppressed by the presence in the reaction mixture of at least one salt of a weak organic acid with a biocompatible cation. By the term "weak organic acid" is meant an organic acid with a pKa in the range 3 to 7. By the term "biocompatible cation" is meant a positively charged counterion which forms a salt with an ionised, negatively charged group, where said positively charged counterion is also non-toxic and hence suitable for administration to the mammalian body, especially the human body. Examples of suitable biocompatible cations include: the alkali metals sodium or potassium; the alkaline earth metals calcium and magnesium; and the ammonium ion. Preferred biocompatible cations are sodium and potassium, most preferably sodium.

Suitable such weak organic acids are acetic acid, citric acid, tartaric acid, gluconic acid, glucoheptonic acid, benzoic acid, phenols or phosphonic acids. Hence, suitable salts are acetates, citrates, tartrates, gluconates, glucoheptonates, benzoates, phenolates or phosphonates, preferably acetates. The acetate salt is preferably sodium or potassium acetate, most preferably sodium acetate. An additional advantage of using these salts is

that the acid anion complexes weakly to the technetium (such as tartrate, gluconate or citrate), and is displaced by the aza-diaminedioxime ligand in a ligand exchange or transchelation process. Such conditions are useful to help suppress undesirable side reactions such as hydrolysis of the technetium ion. A suitable concentration to use for the salt of the weak organic acid is in the range 1 to 100 $\mu\text{mol/ml}$, preferably in the range 5 to 50 $\mu\text{mol/ml}$.

Applicants have further found that the addition of a radioprotectant to the reaction mixture not only stabilises the ^{99}Tc complex product once formed, but unexpectedly accelerates the conversion of the transient ^{99}Tc complexes to the desired thermodynamic technetium complex product. This has the advantage that shorter heating times and/or lower heating temperatures can be applied. This may be particularly advantageous when the biological targeting moiety is heat-sensitive to at least some degree. By the term "radioprotectant" is meant a compound which inhibits degradation reactions, such as redox processes, by trapping highly-reactive free radicals, such as oxygen-containing free radicals arising from the radiolysis of water.

The radioprotectants of the present invention are suitably chosen from: a source of sterile oxygen or air; ascorbic acid; *para*-aminobenzoic acid (ie. 4-aminobenzoic acid or PABA); gentisic acid (ie. 2,5-dihydroxybenzoic acid) and salts of such acids with a biocompatible cation, as defined above. Preferred radioprotectants are ascorbic acid and *para*-aminobenzoic acid, or salts thereof with a biocompatible cation. Especially preferred radioprotectants are *para*-aminobenzoic acid and salts thereof with a biocompatible cation, ideally sodium *para*-aminobenzoate. The radioprotectants of the present invention are commercially available to a pharmaceutical grade specification.

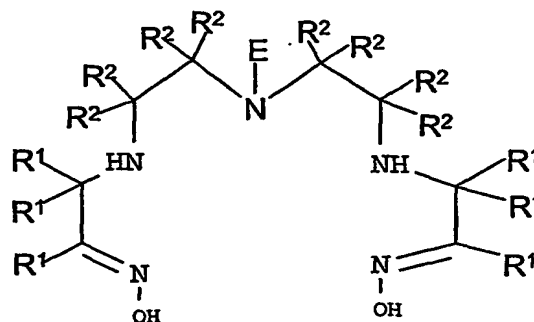
The present inventors have also established that the levels of undesirable lipophilic ^{99}Tc complexes are influenced by the choice of pharmaceutical grade vial closure employed. Such closures typically comprise bromobutyl, chlorobutyl or butyl rubbers, or mixtures thereof, and is believed that volatile leached materials (e.g. hindered phenolic antioxidant or phenolic resin by-products) from the synthetic rubber can react with the aza-

diaminedioxime chelate conjugate to give varying levels of lipophilic ^{99m}Tc complexes. It is believed that most types of closure exhibit this problem, but to varying degrees – hence the choice of closure is also a factor in helping to suppress the levels of lipophilic ^{99m}Tc complexes.

5

The preparation of the technetium complex composition of the present invention is preferably carried out using a non-radioactive kit, as described in the third embodiment of the invention (below).

- 10 The aza-diaminedioxime chelator conjugates used in the preparation of the technetium complex compositions of the present invention can be prepared by reaction of a bifunctional chelate of Formula III with the biological targeting moiety (Z):



Formula III

15

where:

each R^1 and R^2 is independently an R group;

E is $-(A)_n$ -J

where: J is a functional group suitable for conjugation to Z;

$-(A)_n$ - is a linker group where each A is independently

20

$-\text{CO}-$, $-\text{CR}_2-$, $-\text{CR}=\text{CR}-$, $-\text{C}\equiv\text{C}-$, $-\text{CR}_2\text{CO}_2-$, $-\text{CO}_2\text{CR}_2-$, $-\text{NR}-$,

$-\text{NRCO}-$, $-\text{CONR}-$, $-\text{NR}(\text{C}=\text{O})\text{NR}-$, $-\text{NR}(\text{C}=\text{S})\text{NR}-$, $-\text{SO}_2\text{NR}-$,

$-\text{NRSO}_2-$, $-\text{CR}_2\text{OCR}_2-$, $-\text{CR}_2\text{SCR}_2-$, $-\text{CR}_2\text{NRCR}_2-$, a C_{4-8}

cycloheteroalkylene group, a C_{4-8} cycloalkylene group, a C_{5-12}

arylene group, or a C_{3-12} heteroarylene group or a

25

polyalkyleneglycol, polylactic acid or polyglycolic acid moiety;

n is an integer of value 0 to 10;

each R group is independently H or C₁₋₁₀ alkyl, C₃₋₁₀ alkylaryl, C₂₋₁₀ alkoxyalkyl, C₁₋₁₀ hydroxyalkyl, C₁₋₁₀ fluoroalkyl, or 2 or more R groups, together with the atoms to which they are attached form a carbocyclic, heterocyclic, saturated or unsaturated ring.

5

By the term "functional group suitable for conjugation" is meant a functional group which will react with a corresponding functional group of Z (typically an amine, carboxyl or thiol group) to chemically link the aza-diamine dioxime chelator to Z. Preferred such functional groups suitable for conjugation are: -NR⁵R⁶, -CO₂M, -NCS, -NCO, -SM¹, -

10 OM¹, maleimide or acrylamide, where:

R⁵ and R⁶ are independently an R group or P^G;

M is H, a biocompatible cation, P^G or an active ester;

M¹ is H or P^G; and P^G is a protecting group.

The term "biocompatible cation" is as defined above.

15

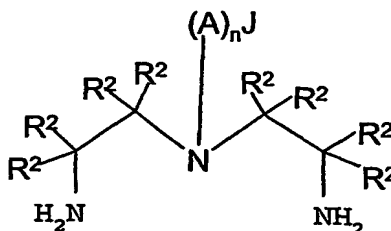
By the term "protecting group" is meant a group which inhibits or suppresses undesirable chemical reactions, but which is designed to be sufficiently reactive that it may be cleaved from the functional group in question under mild conditions that do not chemically modify the rest of the molecule. After deprotection the group in question may be used to conjugate the bifunctional chelate of Formula III to the biological targeting moiety (Z). Protecting groups are well known to those skilled in the art and are suitably chosen from, when J is -NR⁵R⁶: Boc (where Boc is *tert*-butyloxycarbonyl), Fmoc (where Fmoc is fluorenylmethoxycarbonyl), trifluoroacetyl, allyloxycarbonyl, Dde [i.e. 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] or Npys (i.e. 3-nitro-2-pyridine sulfonyl); and when J is -CO₂P^G: methyl ester, *tert*-butyl ester, benzyl ester. When J is -OP^G, suitable protecting groups are: acetyl, benzoyl, trityl or tetrabutyltrimethylsilyl. When J is -SP^G, suitable protecting groups are: trityl and 4-methoxybenzyl. The use of further protecting groups is described in 'Protective Groups in Organic Synthesis', Theodor W. Greene and Peter G. M. Wuts, (John Wiley & Sons, 1991).

30

By the term "active ester" is meant an ester derivative of the carboxylic acid which is designed to be a better leaving group, and hence permit more facile reaction with nucleophiles present on the biological targeting moiety such as amines. Examples of suitable active esters are: N-hydroxysuccinimide (NHS), pentafluorophenol, pentafluorothiophenol, *para*-nitrophenol, hydroxybenzotriazole and PyBOP (ie. benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate).

The bifunctional chelates of Formula III can be conjugated to the biological targeting moiety (Z) as described by S.Liu *et al* [Chem.Rev., 99, 2235-2268 (1999)]. Thus, eg. amine-functionalised aza-diamine dioxime chelators of Formula III (ie. J = -NR⁵R⁶) can be conjugated to the carboxyl group(s) of the biological targeting moiety (Z), *via* amide bonds. This coupling can be carried out directly (eg. using solid phase peptide synthesis), or *via* appropriate intermediates as is known in the art, such as activated esters of the carboxyl group of the biological targeting moiety. Alternatively, the pendant amine group of the bifunctional chelator can first be converted to an isothiocyanate (-NCS) or isocyanate (-NCO) group, which permit conjugation to amine-containing biological targeting moieties, *via* the formation of thiourea and urea linkages respectively. Alternatively, the pendant amine group of the bifunctional aza-diamine dioxime chelator can be reacted with one acid function of a diacid to introduce a terminal carboxyl group *via* a linker group. A bifunctional chelator bearing a carboxyl function (ie. J = -CO₂M) can be used in a similar manner to couple directly to amine-containing biological targeting moieties *via* an amide bond. The bifunctional chelate may also bear a group designed to react with thiol groups on the biological targeting moiety to form stable thioether linkages. Examples of such groups are maleimides (which may be prepared by reaction of maleic anhydride with the corresponding amine, followed by heating with acetic anhydride), and acrylamides (which may be prepared by reaction of acrylyl chloride with the amine).

The bifunctional aza-diaminedioxime chelators of the present invention may suitably be prepared by alkylation of a compound of Formula IV:



Formula IV

where A, J, R^2 and n are as defined for Formula III above,
with either:

- 5 (i) the appropriate chloronitroso derivative $Cl-C(R^1)_2-C(=NO)R^1$;
- (ii) an alpha-chloro oxime of formula $Cl-C(R^1)_2-C(=NOH)R^1$;
- (iii) an alpha-bromoketone of formula $Br-C(R^1)_2-C(=O)R^1$ followed by conversion of the diaminediketone product to the diaminedioxime with hydroxylamine.

10 Route (i) is described by S. Jurisson *et al* [Inorg. Chem., 26, 3576-82 (1987)]. Chloronitroso compounds can be obtained by treatment of the appropriate alkene with nitrosyl chloride (NOCl). Further synthetic details of chloronitroso compounds are given by: Ramalingam, K. *et al* Synth. Commun. (1995) 25(5) 743-52; Glaser *et al* J. Org. Chem. (1996), 61(3), 1047-48; Clapp, Leallyn B.; *et al* J. Org Chem. (1971), 36(8) 1169-
15 70; Saito, Giulichi *et al* Shizen Kagaku (1995), 47, 41-9 and Schulz, Manfred Z. Chem (1981), 21(11), 404-5. Route (iii) is described by Nowotnik *et al* [Tetrahedron, 50(29), p.8617-8632 (1994)]. Alpha-chloro-oximes can be obtained by oximation of the corresponding alpha-chloro-ketone or aldehyde, which are commercially available. Alpha-bromoketones are commercially available.

20

In a second aspect, the present invention provides radiopharmaceuticals which comprise the above aza-diaminedioxime technetium complex compositions in a sterile form suitable for human administration. Such radiopharmaceuticals are suitably supplied in a container which is provided with a seal which is suitable for single or multiple puncturing
25 with a hypodermic needle (e.g. a crimped-on septum seal closure) whilst maintaining sterile integrity. Such containers may contain single or multiple patient doses. Preferred

multiple dose containers comprise a single bulk vial (e.g. of 10 to 100 cm³ volume) which contains multiple patient doses, whereby single patient doses can thus be withdrawn into clinical grade syringes at various time intervals during the viable lifetime of the preparation to suit the clinical situation.

5

The radiopharmaceuticals of the present invention may also be supplied in pre-filled syringes. Such pre-filled syringes are designed to contain a single human dose, which is in a form wherein the dose can be administered to the patient directly from the syringe. Such pre-filled syringes are suitably prepared by aseptic manufacture, so that the product is in sterile form. The syringe which is pre-filled is therefore preferably a disposable or other syringe which is suitable for clinical use, and hence which maintains the sterile integrity of the radiopharmaceutical. The pre-filled syringe containing the radiopharmaceutical may advantageously be provided with a syringe shield to protect the operator from radioactive dose. Suitable such radiopharmaceutical syringe shields are known in the art and preferably comprise either lead or tungsten. Such pre-filled syringes are preferably shipped to the customer already fitted with a syringe shield, and packaged within a container which provides further radiation shielding, so that the external radiation dose at the exterior of the package is minimised during transport from the manufacturer to the customer.

20

A ^{99m}Tc radioactivity content suitable for a ^{99m}Tc diagnostic imaging radiopharmaceutical is in the range 180 to 1500 MBq (3.5 to 42 mCi), depending on the site to be imaged *in vivo*, the uptake and the target to background ratio. For heart imaging with a ^{99m}Tc radiopharmaceutical, ca. 1110 MBq (30 mCi) may be used for a stress study, and ca. 350 MBq (10 mCi) for a rest study. For thrombus imaging with a ^{99m}Tc radiopharmaceutical ca. 750 MBq (21 mCi) would be suitable.

25

In a third aspect, the present invention provides non-radioactive kits for the preparation of the ^xTc radiopharmaceutical compositions of the present invention. Such kits are designed to give sterile radiopharmaceutical products suitable for human administration, e.g. *via* direct injection into the bloodstream. For ^{99m}Tc, the kit is preferably lyophilised

30

and is designed to be reconstituted with sterile ^{99m}Tc -pertechnetate (TcO_4^-) from a ^{99m}Tc radioisotope generator to give a solution suitable for human administration without further manipulation. Suitable kits comprise a container (eg. a septum-sealed vial) containing the uncomplexed aza-diaminedioxime ligand of Formula (I) or (II), together with a
5 pharmaceutically acceptable reducing agent such as sodium dithionite, sodium bisulphite, ascorbic acid, formamidine sulphinic acid, stannous ion, Fe(II) or Cu(I) ; together with at least one salt of a weak organic acid with a biocompatible cation as defined above. The salt of the weak organic acid functions to suppress the formation of undesirable lipophilic ^{99m}Tc complexes (as described above).

10

The non-radioactive kits may optionally further comprise a second, weak organic acid or salt thereof with a biocompatible cation, which functions as a transchelator. The transchelator is a compound which reacts rapidly to form a weak complex with technetium, then is displaced by the aza-diaminedioxime. This minimises the risk of
15 formation of reduced hydrolysed technetium (RHT) due to rapid reduction of pertechnetate competing with technetium complexation. Suitable such transchelators are the weak organic acids and salts thereof described above, preferably tartrates, gluconates, glucoheptonates, benzoates, or phosphonates, preferably phosphonates, most especially diphosphonates. A preferred such transchelator is MDP, ie. methylenediphosphonic acid,
20 or a salt thereof with a biocompatible cation.

25

As an alternative to use of the ligand in free form, the kit may optionally contain a metal complex of the aza-diaminedioxime ligand which, upon addition of the technetium, undergoes transmetallation (i.e. ligand exchange) giving the desired product. Suitable
such complexes for transmetallation are copper or zinc complexes.

30

The pharmaceutically acceptable reducing agent used in the kit is preferably a stannous salt such as stannous chloride, stannous fluoride or stannous tartrate, and may be in either anhydrous or hydrated form. The stannous salt is preferably stannous chloride or
stannous fluoride.

The non-radioactive kits may optionally further comprise additional components such as a radioprotectant, antimicrobial preservative, pH-adjusting agent, or filler. The term "radioprotectant" is as defined above. By the term "antimicrobial preservative" is meant an agent which inhibits the growth of potentially harmful micro-organisms such as bacteria, yeasts or moulds. The antimicrobial preservative may also exhibit some bactericidal properties, depending on the dose. The main role of the antimicrobial preservative(s) of the present invention is to inhibit the growth of any such micro-organism in the ^{99m}Tc radiopharmaceutical composition post-reconstitution, ie. in the radioactive diagnostic product itself. The antimicrobial preservative may, however, also optionally be used to inhibit the growth of potentially harmful micro-organisms in one or more components of the non-radioactive kit of the present invention prior to reconstitution. Suitable antimicrobial preservative(s) include: the parabens, ie. methyl, ethyl, propyl or butyl paraben or mixtures thereof; benzyl alcohol; phenol; cresol; cetrimide and thiomersal. Preferred antimicrobial preservative(s) are the parabens.

The term "pH-adjusting agent" means a compound or mixture of compounds used to ensure that the pH of the reconstituted kit is within acceptable limits (approximately pH 4.0 to 10.5) for human or mammalian administration. Suitable such pH-adjusting agents include pharmaceutically acceptable buffers, such as tricine, phosphate or TRIS [ie. *tris*(hydroxymethyl)aminomethane], and pharmaceutically acceptable bases such as sodium carbonate, sodium bicarbonate or mixtures thereof. For the aza-diaminedioximes of the present invention, it is preferred that the pH-adjusting agent comprises sodium bicarbonate.

When the biological targeting moiety is a peptide fragment of α_2 -antiplasmin, a preferred kit formulation comprises: the ligand of Formula (I), stannous reductant, an acetate salt of a biocompatible cation, a diphosphonic acid transchelator plus a pH-adjusting agent. A preferred such kit comprises: the ligand of Formula (II); stannous chloride; sodium acetate; MDP or a biocompatible salt thereof; a radioprotectant, especially PABA or a biocompatible salt thereof, most especially the sodium salt of PABA; and sodium bicarbonate as the pH-adjusting agent. A most preferred such kit further comprises the

ligand of Formula II, where each R^1 is CH_3 , $(A)_p$ is NH and Z is Ac-Asn-Gln-Glu-Gln-Val-Ser-Pro-Xaa-Thr-Leu-Leu-Lys-Gly-, where Xaa is Tyr or I-Tyr, and Ac is N-acetyl.

In a fourth aspect, the present invention provides a method of diagnostic imaging of thrombi using the radiopharmaceuticals of the present invention, where the biological targeting molecule is a 3 to 20-mer peptide fragment of α_2 -antiplasmin. Preferably, the peptide fragment of α_2 -antiplasmin comprises the sequence Asn-Gln-Glu-Gln. Most preferably the peptide fragment of α_2 -antiplasmin comprises the sequence Asn-Gln-Glu-Gln-Val-Ser-Pro-Xaa-Thr-Leu-Leu-Lys-Gly, where Xaa is as defined above. Pulmonary emboli (PE) are composed of significant amounts of fibrin, which is cross-linked and stabilised by the action of Factor XIIIa. Both fibrin and Factor XIIIa are generated from non-active precursors specifically at sites of thrombosis. The peptide fragments of α_2 -antiplasmin of the present invention are potent substrates for Factor XIIIa, and are thus covalently bound to fibrin within pulmonary emboli *via* the action of this enzyme. The ^{99m}Tc -complexes of the aza-diaminedioxime α_2 -antiplasmin peptide fragment conjugates of the present invention are thus selectively taken up at the site of the embolus *in vivo*, giving positive uptake or "hot-spot" imaging for such sites relative to normal tissue. Similar logic applies to other types of thrombi *in vivo* (eg. deep vein thrombosis or dvt), since Factor XIIIa functions in a similar way. Hence, the Factor XIIIa substrate conjugates of the present invention are useful for the imaging of thrombi *in vivo*, especially pulmonary emboli and deep vein thrombosis.

The invention is illustrated by the non-limiting Examples detailed below.

Example 1 describes the synthesis of Compound 1, which is an aza-diaminedioxime chelator of the invention.

Example 2 describes the synthesis of the protected form of a preferred peptide targeting molecule, which is a peptide fragment of α_2 -antiplasmin for targeting thrombi.

Example 3 describes the synthesis of Compound 3, which is a conjugate of the aza-diaminedioxime chelator of Example 1 with the targeting peptide of Example 2. Example 3 also describes the syntheses of Compounds 4 to 7.

Example 4 is a comparative Example showing the ^{99m}Tc radiolabelling of Compound 3 as taught in the prior art.

Example 5 describes the HPLC systems used in the present invention. System J is the one which gives an apparently acceptable RCP, but lipophilic ^{99m}Tc complexes are retained on the column (ie. they have too great an affinity for the stationary phase when up to 40% acetonitrile is used in the mobile phase). System H has an elution gradient which includes more organic solvent in the mobile phase (up to 90% acetonitrile), and hence successfully elutes the lipophilic technetium complexes from the column, so that they are analysed. The 4% overestimate of the purity using System J is due to undetected

lipophilic ^{99m}Tc complexes (see Figure 3). Example 10 and Figure 1 show that such levels of lipophilic ^{99m}Tc complexes have a significant effect on the liver uptake *in vivo*.

Example 6 describes the preparation of lyophilised kits of the present invention.

Formulation 82/6 is preferred, since there is less tendency for the freeze-dried cake to collapse during lyophilisation. Formulation 82/6 is also more robust to aged ^{99m}Tc

generator eluate (ie. up to 6 hours after elution of the generator), giving satisfactory initial RCP and good post reconstitution stability of the preparation.

Example 7 describes the reconstitution of the lyophilised kits to give ^{99m}Tc complex compositions of the present invention.

Example 8 shows that lysine (K)-containing peptides, ie. having amine groups

(Compounds 3 and 4) show significant levels of lipophilic technetium complexes. When these are replaced with non-amine containing amino acids as per Compound 5, the level of lipophilic complexes is much reduced. This is also shown by the benzamide derivative (Compound 6).

Example 9 shows the effect of the use of a radioprotectant on the ^{99m}Tc complex compositions of the present invention.

Example 10 demonstrates the effect of vial closure on the ^{99m}Tc complex composition.

Example 11 shows the effect of the lipophilic ^{99m}Tc complex content on the biodistribution of a ^{99m}Tc complex composition. The variation in lipophilic impurities caused a significant increase ($p < 0.05$) in blood retention of radioactivity at 1 hour p.i. (1.88 to 3.26%) with a concomitant trend towards a decrease in urinary output (63.07 to 51.04%) although not statistically significant. Radioactivity present in the liver was also

significantly increased ($p < 0.05$) from 2.24% to 10.24%. Thus, increasing levels of lipophilic impurities leads to significantly increased retention of ^{99m}Tc -Compound 3 in the blood and significantly higher uptake in the liver. This in turn caused a trend towards decreased urinary excretion.

- 5 Example 12 shows the effect of a weak organic acid salt on the lipophilic technetium complex content and resulting biodistribution. The addition of sodium acetate trihydrate to the formulation of ^{99m}Tc -Compound 3 is shown to increase blood clearance and decrease liver uptake, as would be expected due to the suppressed content of lipophilic ^{99m}Tc complexes present. The diagnostic imaging potential of the composition is thus
- 10 significantly improved. Uptake of the agent into heart and lung is negligible and hence the agent is potentially useful for lung PE imaging due to the satisfactory low background uptake.

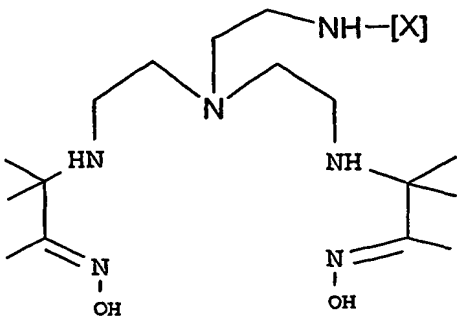
- Example 13 shows that an improved ^xTc complex composition of the present invention still exhibits useful clot uptake, ie. the biological targeting activity is retained. As
- 15 demonstrated from Example 11 and Figure 1, the major biological characteristic resulting from the presence of the lipophilic ^{99m}Tc complexes is an increase in the %id in the liver.

Figure 1 shows the effect of lipophilic ^{99m}Tc complex content in the composition on the liver uptake.

- 20 Figure 2 shows a representative HPLC trace of a aza-diaminedioxime ^{99m}Tc complex composition showing the various species described in the present invention – Figure 2A is normal full scale peak display, and Figure 2B is expanded scale.

- Figure 3 compares the HPLC traces for the same aza-diaminedioxime ^{99m}Tc complex preparation using two different systems - System J and System H. The lipophilic ^xTc
- 25 complexes are detected using System H (at a level of ca. 5% of the radiolabelled material present), but are undetected using System J.

Table 1

	
Compound	X
1.	H
3.	-GKLLT(I-Y)PSVQEQN-Ac
4.	-CO(CH ₂) ₂ CO ₂ -VHHQKLVFF-NH ₂
5.	-GALLTYPVAQAN-Ac
6.	-COPh
7.	-CO(CH ₂) ₂ CO ₂ H

Where: Ac is acetyl.

5 **Example 1: Synthesis of 3,3,11,11-tetramethyl-7-(2-aminoethyl)-4,7,10-triazatridecane-2,12-dionedioxime (Compound 1 or Pn216).**

To a solution of *tris*(2-aminoethyl)amine (1ml, 6.68mmol) in acetonitrile (10ml) was added sodium bicarbonate (1.12g, 13.36mmol, 2eq). A solution of 3-chloro-3-methyl-2-nitrosobutane [R.K.Murmann, J.Am.Chem.Soc, 79, 521-526(1957); 1.359g, 10.02mmol, 1.5eq] in dry acetonitrile (5ml) was added slowly. The reaction mixture was left to stir at room temperature for 3 days, and then filtered. The residue was washed well with acetonitrile, and the filtrate evaporated. The crude product was then purified by RP-HPLC (column: Hamilton PRP-1; gradient: 0 to 100%B in 20 min; where Eluent A is 2% aqueous NH₃ and Eluent B is acetonitrile, at a flow rate of 3ml/min) to afford Compound 1 (164mg, 7%).

δ_H (CD₃OD, 300MHz): 2.77 (2H, t, *J* 6Hz, CH₂NH₂), 2.50-2.58 (10H, m, H₂NCH₂CH₂N(CH₂CH₂NH)₂), 1.85 (6H, s, 2 x CH₃C=N), 1.23 (12H, s, 2 x (CH₃)₂CNH).

Example 2: Synthesis of the Peptide Ac-NQEQVSPY(3I)TLLKG (Compound 2).

The protected peptide Ac-Asn(Trt)-Gln(Trt)-Glu(OtBu)-Gln(Trt)-Val-Ser(tBu)-Pro-Tyr(3I)-Thr(tBu)-Leu-Leu-Lys(Boc)-Gly-OH was assembled on a 2-chlorotrityl resin by anchoring Fmoc-Gly- to the resin, and then successive deprotections/coupling
5 cycles with the appropriate protected amino acids and the coupling reagents DCC and HOBt. Solid phase peptide synthesis is described in as described in P. Lloyd-Williams, F. Albericio and E. Girald; *Chemical Approaches to the Synthesis of Peptides and Proteins*, CRC Press, 1997. The terminal asparagine was acetylated, cleaved from the resin using 0.5 % TFA and Compound 2 used without further purification as the
10 trifluoroacetate salt.

Example 3: Synthesis of Compounds 3 to 7.

The protected Ac-NQEQVSPY(3I)TLLKG peptide (Compound 2) was cleaved from the solid phase resin as described in Example 2, and then coupled with Compound 1 in
15 solution using PyBOP (benzotriazole-1-yl-oxytris-pyrrolidino-phosphonium hexafluorophosphate) and HOBt (1-hydroxybenzotriazole) as the coupling agents. Compound 3 was obtained by deprotection in reagent K [reagent K is 82.5% TFA, 5% phenol, 5% processed water, 5% thioanisole, 2.5% ethanedithiol (EDT)]. The crude
20 conjugate was first purified by RP-HPLC using TFA followed by a second purification and salt exchange with acetic acid, lyophilisation, filtration with a 0.22µ filter and a final lyophilisation to give Compound 3.

Molecular weight by MS 1970 ± 1 Daltons.

The peptides used in Compounds 4 and 5 were prepared in a similar manner. Compound
25 7 was prepared by derivatisation of Compound 1 (Pn216) with glutaric anhydride in DMF. Compound 6 was prepared by reaction of Compound 1 (Pn216) with benzoic anhydride in acetonitrile, in the presence of triethylamine.

Example 4: Tc-99m Radiolabelling of Compound 3 [Comparative Example].

30 A 0.1ml aliquot of Compound 3 dissolved in H₂O (1mg/ml) was transferred to a nitrogen-filled 10ml glass vial together with deoxygenated saline (0.9% w/v, 1ml) and 0.035ml

aqueous NaOH (0.1M). To this solution was added technetium generator eluate (1ml, approx. 0.4GBq) and then aqueous stannous chloride solution (0.1ml, ca.10 μ g). The labelling pH was 9.0-10.0. Vials were incubated at ambient laboratory temperature (15-25°C) for 30 minutes to effect labelling. The resulting preparation was either diluted to the desired radioactive concentration or HPLC purification was performed (System B) to remove unlabelled starting material and radioactive impurities prior to testing. After purification the organic solvent was removed *in vacuo* and the sample was redissolved in about 5ml 0.1M phosphate buffer pH 7.4 to give a working concentration of 6-9MBq/ml. Radiochemical purity was assessed before use by the thin layer chromatography (TLC) system described below:

- i) ITLC SG 2cm x 20cm eluted with 0.9% w/v saline
- ii) Whatman No.1 2cm x 20cm eluted with 50:50 v/v acetonitrile:H₂O

The labelled substrates remain at, or close to, the origin in TLC system (i) and move close to the solvent front in system (ii).

Example 5: HPLC Systems.

Flow Rate: 1ml/min in all systems, and TFA = trifluoroacetic acid.

System A

Column Waters C18 250x4.5mm. Particle size 4 microns
Gradient: Elution Profile 10-60%B in 25 min.
Eluent A: 0.1% aqueous TFA
Eluent B: 0.1% TFA in acetonitrile.

System B.

Column Waters Novapak C18 150x3.9mm. Particle size 4 microns
Gradient: Elution Profile 0-100%B in 22 min.
Eluent A: 0.1% aqueous TFA
Eluent B: 0.1% TFA in acetonitrile

System H.

Column Phenomenex C18 (2) Luna 25x0.46cm, 5 μ m;
Gradient: Elution Profile 16-40%B from 0 to 20min, 40-90%B from 20-22min, 90%B 22-30min, then 90-16%B from 30-31min. The column is then re-equilibrated in 16%B for 14min.
Eluent A: 0.05 % TFA in water.

Eluent B: 0.04 % TFA in acetonitrile.

System J.

Column Phenomenex C18 (2) Luna 25 x 4.6 cm, 5 μ m;

5 Gradient: Elution profile 16 – 40 % B from 0 to 20 minutes, 40 – 16% B from 20 – 22 minutes. The column is re-equilibrated in 16 % B for 8 minutes.

Eluent A: 0.05 % TFA in water

Eluent B: 0.05 % TFA in acetonitrile.

- 10 Analysis by HPLC System J (Example 5) of a reconstituted freeze-dried kit (Lot 58, Example 6) showed an RCP of 94% using System J (prior art), but an RCP of 90% using System H (present invention) – see Figure 3.

15 **Example 6: Preparation of Lyophilised Kits.**

Approximately 90% of the total volume of water for injection (WFI) was placed in the preparation vessel and deoxygenated by nitrogen purging. Methylene diphosphonic acid, stannous chloride dihydrate, Compound 3 (as the acetate salt), sodium acetate trihydrate and *p*-aminobenzoic acid, sodium salt were added in turn allowing each one to dissolve
20 whilst nitrogen purging was continued. The nitrogen purging of the solution was replaced with a nitrogen flow over the solution headspace. Sodium hydrogen carbonate and sodium carbonate anhydrous, or sodium hydrogen carbonate alone were then added, and allowed to dissolve. The bulk solution was then adjusted to 100% of the final volume (~5 litre) with deoxygenated WFI. The bulk solution was then filtered through a sterile 0.2
25 μ m filter into the filling vessel. The headspace of the filling vessel was purged with sterile filtered (0.2 μ m) nitrogen or argon for the duration of filling operation. Aliquots of 1.0 ml were dispensed aseptically into vials. The vials were half-stoppered with closures A, B or C and transferred onto pre-chilled freeze-dryer shelves. The vials were then lyophilised, back-filled with sterile filtered (0.2 μ m) nitrogen gas, stoppered and sealed.

Table 2: Kit formulations.

Lot	Cmpd 3 (μg)	SnCl_2 (μg)	MDP (μg)	NaHCO_3 (μg)	Na_2CO_3 (μg)	NaOAc , $3\text{H}_2\text{O}$ (μg)	Na- pABA (μg)	Closure
33*	50	10	0**	2520	350	0	0	A
58	50	30	90	2520	350	0	0	A
63	50	30	90	5040	700	0	0	A
65	50	30	90	2520	350	4150	0	A
70	100	30	90	1260	175	6600	0	A
86	100	30	90	1260	175	6600	200	B
87	100	30	90	1260	175	6600	0	B
88	100	30***	90	1260	175	6600	0	A
94	100	30***	90	1260	175	6600	50	B
98	100	30***	90	1320	98	6600	50	A
82/4	100	30***	90	1320	98	6600	50	A
82/6	100	30***	90	3300	0	530	200	C

where Closure A = PH701/45 AS red-brown; closure B = 4023/50 1083 grey; C = 4432/50 1178 grey.

* lot 33 contained 2 mg NaCl as filler,

5 ** lot 33 contained 25 μg Na-tartrate dihydrate as a transchelator,

*** lots 86, 87, 88, 94, 98 and formulation 82/4 used 36 μg $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ equivalent to 30 μg anhydrous SnCl_2

10 **Example 7: Preparation of $^{99\text{m}}\text{Tc}$ Complexes from Lyophilised Kits.**

The freeze-dried kits of Example 6 were reconstituted with $^{99\text{m}}\text{Tc}$ -pertechnetate (2 – 8 ml of technetium generator eluate; 0.5 – 2.5 GBq). The solution was heated in a water bath at 60 °C for 10 minutes and then allowed to stand at room temperature. Radiochemical purity was determined by the HPLC method described in Example 5 System H (see

15 Figure 2) and the RHT was determined by the ITLC method described below:

ITLC SG 2 cm x 20 cm eluted with a 50:50 (v/v) methanol: ammonium acetate solution (1M). The ligand based radiolabelled species move to the solvent front. RHT is retained at the origin.

The solutions were analysed by HPLC System H and the results are given in Table 3 below, where the %RCP is the radiochemical purity of the thermodynamic ^{99m}Tc complex.

5 **Table 3: HPLC analyses of ^{99m}Tc Complexes.**

Lot	% RCP	% ^{99m}Tc -Lipophilics
33*	80	7
58	88 - 92	3 - 5
63	70 - 85†	6 - 11
65	78 - 91†	1.0 - 1.5
70	80 - 95†	1.0 - 1.5
88	90 - 94	1.0 - 2.0

† variable RCP believed to be due to variations in heating.

- 10 **Example 8: Effect of Targeting Molecule on ^{99m}Tc Lipophilic Complex Content.** Freeze-dried kit preparation were prepared according to Example 6, for various targeting molecules (Z), using the Formulation of Lot 63 (Example 6). The results are shown in Table 4 for testing of three vials from the batch after reconstitution and analysis as per Example 7:

15

Table 4: Effect of Z on ^{99m}Tc Lipophilic Complex Content

Lot	Compound	% RCP	% ^{99m}Tc -Lipophilics
183	3	89.0	5.8
		80.7	9.3
		79.5	10.2
178	4	86.4	4.6
		89.6	2.2
		84.7	3.4
180	5	84.3	0
		85.2	0.7
		84.8	0.5
182	6	94.5	0.2
		93.7	0.1
		93.7	0.2

Example 9: Effect of Radioprotectant on ^{99m}Tc Complex Preparations.

The effect of the radioprotectant sodium *para*-aminobenzoate (Na-PABA) on the purity of the ^{99m}Tc complex preparations was studied – both in solution, and as freeze-dried kit preparations. All preparations were made in 10R vials with 1083 4023/50 Grey closures, to a final radioactive concentration (RAC) of 0.5 GBq/ml. The preparations were analysed at various times post-reconstitution (PR) using HPLC System H, and the results are given in Table 5 as percentages of the radioactive composition.

Table 5: Labelling analysis for PABA-containing preparations.

Lot	Na pABA ($\mu\text{g/vial}$)	Time PR (min)	^{99m}Tc Complex species (%)							
			RHT	$^{99m}\text{TcO}_4^-$	Other Hydrophilic complexes	Kin	Pre-peak	Td	Post-peaks	Lp
87	0	30	0.7	0	0.6	11.1	1.8	86.1	0	0.1
	0	240	0.7	3.5	0.6	4.4	2.1	88.9	0.6	0
	0	480	0.4	7.1	0.7	2.6	2.3	86.5	0.7	0
87 + pABA	25	30	0.5	0.1	0.5	1.9	2.1	93.1	1.8	0
	25	330	0.6	1.6	0.6	1.3	2.2	93.8	0.3	0
93	25	30	0.6	0	0.5	4.3	2.1	92.4	0.3	0.2
	25	300	0.5	1.3	0.5	2.9	2.2	92.2	0.6	0
	25	480	np	4.6	0.7	3	3.1	87.2	0.7	0.1
87 + pABA	50	30	0.8	0	0.4	1.7	2.2	92.3	3.1	0
	50	240	1	0	0.5	0.7	1.8	94.7	2.3	0
	50	480	0.6	0.7	0.5	1.3	2	93.2	2.4	0
94	50	30	0.7	0	0.8	1.6	2.1	95.2	0.2	0.1
	50	300	0.6	0.9	0.4	1.1	1.9	95.1	0.4	0
	50	480	np	1.9	0.9	2.5	1.9	91	0.8	0.2
87 + pABA	100	30	np	0.1	0.8	0.5	1.9	93.2	3.4	0
	100	300	0.7	0	0.4	0.3	2	95.2	2	0
	100	480	0.7	0.7	0.6	0.3	2	94	2.1	0
86	200	30	0.6	0.2	0.3	0.4	2.1	96	0.2	0.6
	200	240	0.9	0	0.5	0.2	2.1	93.3	2.9	0.7
	200	480	0.8	0.6	0.5	0.3	1.8	96.5	0	0.3

Np = analysis not performed,

Td = thermodynamic product (main peak),

Kin = kinetic complexes,

Lp = Lipophilic complexes.

Example 10: Effect of Closures on RCP and ^{99m}Tc Lipophilic Complexes.

The effect of vial closure on radiolabelling was studied for various lyophilised kit formulations. Lyophilised kits produced using two different rubber closures (West Pharmaceutical Services) were reconstituted according to the method set out in Example 7. Differences in RCP and radiolabelled impurities were noted (see Table 6, which shows the average of 10 RCP determinations):

Table 6: Effect of Closure on RCP and ^{99m}Tc Lipophilic Complexes.

Lot	Formulation	Closure	% RCP (mean of 10)	% ^{99m}Tc Lipophilic Complexes
87*	82/1	4023/50 Grey	86.8	0.1
94	82/2	4023/50 Grey	93.9	0.1
88*	82/1	PH701/45AS Red-brown	92.3	1.3
98	82/2	PH701/45AS Red-brown	93.8	1.4

* Heated at 90 °C for 10 minutes in a heater block.

Example 11: Effect of Lipophilic Technetium Complex Content on Biodistribution.

Experiments were carried out in normal male Wistar rats (body weight 150-250g) and dissections performed at 1 hour post intravenous injection of ^{99m}Tc -Compound 3 preparations. The latter were prepared from a lyophilised kit formulation, described as Lot #58 (see Example 6 for details), and were either unheated, or heated for different times at 90 °C, and cooled to ambient before administration. HPLC analysis using System H (Example 5) confirmed the differing levels of lipophilic impurities shown in Table 6 (below). Biodistribution data was calculated for each preparation organ/tissue data was reported for each individual animal and the mean and standard deviation of the group of three. A single factor/one way analysis of variance (ANOVA) test or an unpaired, 2 tailed Student's t-test (2 samples, unequal variance) was applied to this data

using a Microsoft Excel 97 SR-2 spreadsheet package. Results were deemed to be significantly different if the p value was less than 0.05. The results are given in Table 7:

Table 7: Mean % injected dose (with standard deviations) for preparations heated for various lengths of time (0-45 mins @ 90°C).

Preparation (lot #58)	no heat (13008)		10 min (13009)		45 min (13025)	
Lipophilic content	3.1%		5.4%		7.8%	
Organ	Mean	S.D.	Mean	S.D.	Mean	S.D.
Animal weight	204.05	9.16	212.02	5.03	201.72	3.10
Bone	2.40	0.18	0.89	0.20	0.95	0.07
Muscle	2.71	0.32	2.87	0.61	3.62	0.99
Blood	1.88	0.15	2.79	0.45	3.26	0.29
Kidneys	19.59	3.19	18.96	0.93	18.39	1.02
Bladder & Urine	63.07	4.07	58.22	4.57	51.04	8.20
Lung	0.16	0.02	0.17	0.02	0.21	0.06
Liver	2.24	0.09	6.35	0.34	10.24	0.54
Spleen	0.07	0.01	0.15	0.02	0.26	0.07
Stomach	0.17	0.06	0.35	0.33	0.46	0.16
SI, LI & faeces	2.64	1.16	4.04	1.47	6.15	2.95
Heart	0.04	0.00	0.05	0.01	0.06	0.01
Thyroid	0.02	0.00	0.03	0.01	0.02	0.00
Carcass	5.00	0.96	5.12	2.04	5.34	2.51
Injection site	1.01	0.40	0.86	0.46	0.59	0.01

Example 12: Effect of Weak Acid Salt on Lipophilic Technetium Complex Content and Biodistribution.

The changes in biodistribution caused by the addition of sodium acetate trihydrate to the formulation were evaluated by the comparison of lyophilised kit lot numbers 58 and 65.

Kit reconstitution and biodistribution studies were then carried out as for Example 10.

This data was analysed by use of an unpaired, 2 tailed Student's t-test. There was a

significant decrease ($p < 0.05$) in blood retention of radioactivity (2.95 to 2.12%) and also a significant decrease in liver associated radioactivity ($p < 0.05$) from 6.10% to 2.82%.

Table 8: Mean % injected dose (with standard deviations) for formulations with or without sodium acetate trihydrate.

Lot # (Preparation)	58 (15093)		65 (15160)	
Formulation	No acetate		With acetate	
Lipophilic content	3.1%		1.0%	
Organ	Mean	S.D.	Mean	S.D.
Animal weight	179.28	4.86	163.72	12.31
Bone	0.98	0.03	0.70	0.02
Muscle	2.87	0.03	2.90	0.17
Blood	2.95	0.08	2.12	0.02
Kidneys	18.52	0.71	21.37	1.00
Bladder & Urine	61.66	0.85	61.23	2.43
Lung	0.23	0.01	0.16	0.04
Liver	6.10	0.38	2.82	0.09
Spleen	0.16	0.01	0.07	0.00
Stomach	0.15	0.04	0.39	0.40
SI, LI & faeces	2.48	0.43	3.27	1.50
Heart	0.06	0.00	0.06	0.01
Thyroid	0.03	0.00	0.03	0.01
Carcass	3.81	0.26	4.90	0.26
Injection site	0.73	0.02	0.82	0.12

5 Example 13: Comparison of clot uptake in a rat model of venous thromboembolism.

Preparations with known variations in lipophilic $^{*}\text{Tc}$ complexes (Lots 58 and 65 – see Example 7) were studied. Thus, rats (male Wistar, 250-350g) were anaesthetised with 15% urethane. After laparotomy, the vena cava was isolated and freed of surrounding fat tissue. A platinum wire (1.5cm x 0.5mm) was inserted into the inferior vena cava and 5 minutes later 0.4ml of ellagic acid (1.2×10^{-4} M) was injected intravenously through the femoral vein to initiate clot formation. 60 minutes later 0.1ml (50MBq per animal) of either lots 58 or 65 (prepared as per Example 7) were injected *via* the same vein and after a further 60 minutes the animals were sacrificed and the clot removed, weighed and counted. Other tissues e.g. blood, lung, heart, were also dissected and counted. The uptake of tracer into the clot was determined as the relative concentration (cpm/g of clot by dose/g animal) and clot to background tissue. The average weight of the clots formed in this model was around 27mg, n=32, (5-50mg range).

The relative concentration of radioactivity in the clot was 8.01 (n=4, SD=3.33) for Lot 58 and 7.49 (n=4, SD=1.84) for Lot 65.